3-Aminobenzanthrone, a human metabolite of the carcinogenic environmental pollutant 3-nitrobenzanthrone, induces biotransformation enzymes in rat kidney and lung*

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Abbreviations: acetyl-CoA, acetyl coenzyme A; 3-ABA, 3-aminobenzanthrone; Ah, aryl hydrocarbon; COX, cyclooxygenase; c_T, cycle threshold; CYP, cytochrome P450; dA-N^6-ABA, 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone; dG, deoxyguanosine; dG-N^2-ABA, 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone; dG-C8-*N*-ABA, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone; EROD, 7-ethoxyresorufin *O*-deethylation; LPO, lactoperoxidase; MPO, myeloperoxidase; NAT, *N*,*O*-acetyltransferase; 3-NBA, 3-nitrobenzanthrone; *N*-OH-3-ABA, *N*-hydroxy-3-aminobenzanthrone; NQO1, NAD(P)H:quinone oxidoreductase; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; PCR, polymerase chain reaction; RAL, relative adduct labelling; RT, real-time; SDS, sodium dodecyl sulphate; SULT, sulfotransferase; TLC, thin-layer chromatography;

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Abstract

3-Aminobenzanthrone (3-ABA) is the metabolite of the carcinogenic air pollutant 3-nitrobenzanthrone (3-NBA). 3-ABA was investigated for its ability to induce cytochrome P450 1A1 (CYP1A1) and NAD(P)H:quinone oxidoreductase (NQO1) in kidney and lung of rats, and for the influence of such induction on DNA adduct formation by 3-ABA and 3-NBA. NQO1 is the enzyme that reduces 3-NBA to *N*-hydroxy-3-aminobenzanthrone (*N*-OH-3-ABA) and CYP1A enzymes oxidize 3-ABA to the same intermediate. Both compounds activated by cytosolic and and/or microsomal fractions isolated from rat lung, the target organ for 3-NBA carcinogenicity, and kidney, generated the same DNA adduct pattern, consisting of five adducts. When pulmonary cytosols isolated from rats that had been treated i.p. with 40 mg/kg bw of 3-ABA were incubated with 3-NBA, DNA adduct formation was up to 1.7-fold higher than in incubations with cytosols from control animals. This increase corresponded to an increase in protein level and enzymatic activity of NQO1. In contrast, no induction of NQO1 expression by 3-ABA treatment was found in the kidney. Incubations of 3-ABA with renal and pulmonary microsomes of 3-ABA-treated rats led to an increase of up to a 4.5-fold in DNA adduct formation relative to controls. The stimulation of DNA adduct formation correlated with the higher protein expression and activity of CYP1A1 induced by 3-ABA. These results show that by inducing lung and kidney CYP1A1 and NQO1, 3-ABA increases its own enzymatic activation as well as that of the environmental pollutant, 3-NBA, thereby enhancing the genotoxic and carcinogenic potential of both compounds.

1. Introduction

3-Aminobenzanthrone (3-ABA, Fig. 1) is the reduced metabolite of the carcinogenic environmental pollutant, the nitroketone 3-nitrobenzanthrone (3-nitro-*7H*-benz[*de*]anthracen-7-one, 3-NBA, Fig. 1) [1,2]. In recent years 3-NBA has received much attention due to its presence in diesel exhaust and its extremely high mutagenic potency in the Salmonella Ames assay [3-5]. 3-NBA is carcinogenic in rats, causing lung tumours after intratracheal instillation, and it is also a suspected human carcinogen [4-6]. The uptake of 3-NBA in humans has been demonstrated by the detection of its metabolite 3-ABA in urine samples of salt mine workers occupationally exposed to diesel emissions [4]. 3-ABA was also the main metabolite of 3-NBA formed in human fetal bronchial cells and rat lung alveolar type II cells [7]. In addition, 3-ABA was found suitable for dyeing microporous polyethylene films, which are widely used for practical purposes such as separation of liquid mixtures, in particular, as separation membranes in chemical batteries [8], or to fluorescently label phospholipid membranes in the form of its *N*-palmitoyl derivative [9]. This suggests its industrial and/or laboratory utilization, leading to a putative exposure of people. This is a matter of concern, because we have demonstrated the genotoxicity of both 3-NBA and 3-ABA by the detection of specific DNA adducts formed *in vitro* and *in vivo* [10-17]. The predominant DNA adducts formed from 3-NBA and 3-ABA are 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-ABA) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-ABA) and these are most probably responsible for the induction of GC to TA transversion mutations induced by these toxicants [15,17-19].

Recently we have demonstrated the role of rat hepatic, pulmonary and renal NAD(P)H:quinone oxidoreductase (NQO1) in reducing 3-NBA to species that are further activated by *N*,*O*-acetyltransferases (NATs) and sulfotransferases (SULTs) to form DNA adducts [13,20,21]. These enzymes are also the major activation enzymes for 3-NBA in human liver [13]. Cytochromes P450 (CYP) 1A1 and 1A2 are essential for the oxidative activation of 3-ABA in human and rat livers, leading to the same DNA adducts that are formed *in vivo* by 3-ABA or 3-NBA [12]. CYP1A1 is

also an efficient activator of 3-ABA in microsomal fractions from rat kidneys and lungs, while prostaglandin H synthase (cyclooxygenase, COX) plays a minor role in this subcellular fraction [21]. Previous results also indicate that besides microsomal CYP enzymes cytosolic peroxidases might play a role in the oxidative activation of 3-ABA, mainly in extrahepatic tissues such as kidneys and lungs [14,20,21]. In *in-vitro* experiments, mammalian COX, lactoperoxidase (LPO) and myeloperoxidase (MPO) were found to be effective in activating 3-ABA [14] (Fig. 1).

We have already shown that both 3-ABA and 3-NBA induce the major enzymes activating both toxins in rat livers (NQO1, CYP1A) [20], and that 3-NBA is also a potent inducer of these enzymes in rat lungs and kidneys [21]. Since lung is the major target of 3-NBA and its major metabolite 3-ABA is excreted in urine, it was important to see if 3-ABA like 3-NBA induces the activating enzymes in these two organs.

2. Material and Methods

2.1. Chemicals

NADPH, deoxyadenosine 3'-monophospate, deoxyguanosine 3'-monophosphate, acetyl coenzyme A (acetyl-CoA), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), menadione (2-methyl-1,4-naphthoquinone) and calf thymus DNA were from Sigma Chemical Co (St Louis, MO, USA); Sudan I from BDH (Poole, UK); 7-ethoxyresorufin from Fluka Chemie AG (Buchs, Switzerland). Enzymes and chemicals for the ^{32}P-postlabelling assay were obtained from sources described [22]. All these and other chemicals were reagent grade or better.

2.2. Synthesis of 3-NBA and 3-ABA

3-NBA and 3-ABA were synthesized as described [23] and their authenticity was confirmed by UV spectroscopy, electrospray mass spectrometry and high field proton NMR spectroscopy.

2.3. Animal experiments

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with Declaration of Helsinki. Male Wistar rats (100-125 g, n=3 per group) were treated with a single i.p. dose of 0.4, 4 or 40 mg/kg bw of 3-ABA (dissolved in sunflower oil at a concentration of 0.4 or 4 mg/ml). Three control animals received an equal volume of sunflower oil only. Rats were placed in cages in temperature and humidity controlled rooms. Standardised diet and water were provided *ad libitum*. The animals were killed 24 hours after treatment by cervical dislocation. Lungs and kidneys were removed immediately after death and used for isolation of mRNA and for preparation of microsomal and cytosolic fractions.

2.4. Preparation of microsomal and cytosolic fractions

Microsomal and cytosolic fractions were isolated from the lungs and kidneys of rats, either uninduced or pretreated with 3-ABA (see above) as described [24,25].

2.5. Cytosolic incubations

The deaerated and argon-purged incubation mixtures, in a final volume of 750 µl, consisted of 50 mM Tris-HCl buffer (pH 7.4), containing 0.2% Tween 20, cofactors for cytosolic enzymes (1 mM NADPH, 2 mM acetyl-CoA; 100 µM PAPS), pooled lung and kidney cytosolic sample from 3 rats, treated either with vehicle (control) or with 40 mg/kg bw of 3-ABA (1 mg of cytosolic protein), 30 µM 3-NBA (dissolved in 7.5 µl dimethylsulfoxide) and 0.5 mg of calf thymus DNA (2 mM dNp). The reaction was initiated by adding 3-NBA. Incubations with rat cytosols were carried out at 37°C for 3 hr; the cytosol-mediated 3-NBA-derived DNA adduct formation was found to be linear up to 4 hr [13]. Control incubations were carried out either (*i*) without activating system (cytosol), (*ii*) without cofactors (NADPH, acetyl-CoA, PAPS), (*iii*) without DNA or (*iv*) without 3-NBA. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described [13].

Incubations used to evaluate the activation of 3-ABA by peroxidases in rat pulmonary and renal cytosolic samples contained, in a final volume of 750 µl, 50 mM Tris-HCl buffer (pH 7.4), containing 0.2% Tween 20, 100 µM 3-ABA (dissolved in 7.5 µl dimethylsulfoxide), 0.5 mg of calf thymus DNA and 200 µM hydrogen peroxide (H_2O_2).

2.6. Microsomal incubations

Incubation mixtures, in a final volume of 750 µl, consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH or 0.1 mM arachidonic acid, pooled pulmonary and renal microsomal fractions from 3 rats, either control or treated with 40 mg/kg bw of 3-ABA (0.5 mg of microsomal protein), 100 µM 3-ABA or 3-NBA (dissolved in 7.5 µl dimethylsulfoxide) and 0.5 mg of calf thymus DNA. The reaction was initiated by adding 3-ABA or 3-NBA and were carried out at 37°C for 2 hr; microsomal-mediated 3-NBA (3-ABA)-derived DNA adduct formation was found to be linear up to 3 hr [12,26]. Control incubations were carried out either (*i*) without activating system (microsomes), (*ii*) with activating system and 3-ABA, but without DNA or (*iii*) with activating system and DNA but without 3-ABA. After the incubation and extraction with ethyl acetate, DNA

was isolated from the residual water phase by the phenol/chloroform extraction method as described [12].

2.7. ^{32}P-Postlabelling analysis and HPLC analysis of ^{32}P-labelled 3',5'-deoxyribonucleoside bisphosphate adducts

^{32}P-Postlabelling analysis using butanol extraction, thin layer chromatography (TLC) and HPLC was performed as described [15]. Enrichment by butanol extraction has been shown to yield more adduct spots and a better recovery of 3-NBA (3-ABA)-derived DNA adducts than using enrichment by nuclease P1 digestion [10,12]. DNA adduct spots were numbered as reported previously [15]. As reference compounds dAp and dGp (4 µmol/ml) (Sigma) were incubated with 3-NBA (300 µM) activated by xanthine oxidase (1 U/ml) (Sigma) in the presence of hypoxanthine and analysed as described previously [10]. DNA adduct standard samples of 3-NBA, 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone-3'-phosphate (dA3'p-N^6-ABA), 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone-3'-phosphate (dG3'p-N^2-ABA) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone-3'-phosphate (dG3'p-C8-*N*-ABA), were prepared by reacting *N*-acetoxy-3-aminobenzanthrone with dAp or dGp and analysed as described recently [15].

2.8. Isolation of CYP1A1

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA [27], in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles University, Prague, Czech Republic).

2.9. Preparation of antibodies

Leghorn chicken were immunised subcutaneously three times a week with rat recombinant CYP1A1 and human recombinant NQO1 (Sigma) antigens (0.1 mg/animal) emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters. Immunoglobulin fraction was purified from pooled egg yolks using fractionation by polyethylene glycol 6000 [27].

2.10. Estimation of CYP1A1 and NQO1 protein content in microsomes and cytosols of rat lung and kidney

Immunoquantitation of rat pulmonary and renal microsomal CYP1A1 and of cytosolic NQO1 was done by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Samples containing 75 µg microsomal or cytosolic proteins were subjected to electrophoresis on SDS/10% polyacrylamide gels [20,21,27,28]. After migration, proteins were transferred onto polyvinylidene difluoride membranes. Rat CYP1A1 and NQO1 proteins were probed with the chicken polyclonal antibodies as reported elsewhere [20,21,27,28]. The antibodies against rat recombinant CYP1A1 and human NQO1 recognise these enzymes in rat pulmonary and renal microsomes as one protein band. Rat recombinant CYP1A1 (in Supersomes^{TM}, Gentest Corp., Woburn, MA, USA) and human recombinant NQO1 (Sigma Chemical Co, St Louis, MO, USA) were used as positive controls to identify the bands of CYP1A1 in microsomes and NQO1 in cytosols. The antigen-antibody complex was visualised with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as chromogenic substrate [20,21,27,28].

2.11. CYP1A1 and NQO1 enzyme activity assays

The microsomal samples were characterised for CYP1A1 activity using 7-ethoxyresorufin *O*-deethylation (EROD) activity and the oxidation of Sudan I [27,28]. The cytosolic samples were characterised for NQO1 activity, using menadione (2-methyl-1,4-naphthoquinone) as a substrate [24,25]. NQO1 activity was determined by following the oxidation of NADPH spectrophotometrically at 340 nm. The standard assay system contained 25 mM Tris-HCl (pH 7.4), 0.2% Tween 20, 0.07% bovine serum albumin, 400 mM NADPH and 100 mM menadione dissolved in methanol.

2.12. CYP1A1 and NQO1 mRNA content in rat lungs and kidneys

Total RNA was isolated from frozen lungs and kidneys of three untreated rats and three rats pretreated with 40 mg/kg body weight of 3-ABA using Trizol Reagent (Invitrogen, Carlsbad, CA,

USA) according to the procedure supplied by the manufacturer. The quality of isolated RNA was verified by horizontal agarose gel electrophoresis, and RNA quantity was assessed by UV-VIS spectrophotometry on a Carry 300 spectrophotometer (Varian, Palo Alto, CA, USA). RNA samples (1 μg) were reverse transcribed using 200 U of reverse transcriptase per sample with random hexamer primers utilising RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The prepared cDNA was used for real-time (RT) polymerase chain reaction (PCR) performed in RotorGene 2000 (Corbett Research, Sydney, Australia) under the following cycling conditions: incubation at 50ºC for 2 min and initial denaturation at 95ºC for 10 min, then 50 cycles of denaturation at 95ºC for 15 s and annealing at 60ºC for 1 min. Gain was set to 7 and fluorescence was acquired after the elongation step. The PCR reaction mixtures (20 μl) contained 9 μl cDNA diluted 10-times in Milli-Q ultrapure water (Biocel A10, Millipore, Billerica, MA, USA), 10 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 1 μl TaqMan Gene Expression Assay Mix (commercially available unlabelled PCR primers and FAM^{TM} dye-labelled probe for rat *CYP1A1* or *NQO1* as target genes and *β-actin* as reference internal standard gene). Each sample was analysed in two parallel aliquots. Negative controls had the same compositions as samples but cDNA was omitted from the mixture. Data were analysed by the program RotorGene v6 (Corbett Research, Sydney, Australia) and evaluated by comparative cycle threshold (c_T) method for relative quantitation of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then $\Delta\Delta c_T$ was evaluated according to following equations: $\Delta c_T = c_T$ (target) - c_T (internal standard), $\Delta\Delta c_T = \Delta c_{Ttreated} - \Delta c_{Tcontrol}$, where $\Delta c_{Ttreated}$ is Δc_T for treated rats and $\Delta c_{Tcontrol}$ is Δc_T for untreated rats. Δc_T is positive if the target is expressed at a lower level than the internal standard (*β-actin*), and negative if expressed at a higher level. The induction of mRNA expression of studied target genes in pretreated animals was evaluated as $2^{-(\Delta\Delta cT)}$.

3. Results

3.1. The effect of 3-ABA pretreatment on the expression of enzymes activating 3-NBA and 3-ABA

Because CYP1A1 and NQO1 are the essential enzymes activating 3-ABA and 3-NBA in rat lungs and kidneys [24] and in rat and human livers [12,13], we evaluated whether treating rats with 3-ABA influences their expression. Induction of these enzymes has already been found in the livers of rats treated with 3-ABA or the parent nitroketone, 3-NBA [20]. Western blots with chicken polyclonal antibodies raised against rat CYP1A1 showed that the expression of this enzyme was also induced in rat lung and kidney by 3-ABA (Fig. 2), whereby the induction of protein expression was higher in kidney than in lung (3.4-fold *versus* 2.5-fold at 40 mg/kg bw). EROD activity and oxidation of Sudan I, markers of CYP1A1 activity [27,28], were higher in both organs of rats treated with 3-ABA (Table 1). Again, the induction in kidney was higher than in lung (up to 4.8-fold *versus* 2.5-fold at 40 mg/kg bw), although control enzyme activity in lung was only half of that in kidney.

In contrast to CYP1A1, the levels of renal NQO1 protein were not increased by pretreating rats with 3-ABA; on the contrary lower expression levels were found in kidney cytosolic fractions of rats treated with 4 and 40 mg/kg bw of 3-ABA (Fig. 2). In the case of lung, NQO1 protein expression was increased 1.4-fold in rats treated with 40 mg/kg bw of 3-ABA. The increase in NQO1 activities in pulmonary cytosol depended on the administered dose and correlated with protein expression (Table 2 and Fig. 2). Up to a 1.6-fold increase in NQO1 activity measured with menadione as a substrate was found in lung of rats treated with 3-ABA (Table 2).

Besides the evaluation of the effects of 3-ABA on protein levels and enzyme activities of CYP1A1 and NQO1, changes in mRNA expression levels by the compound were also investigated. The relative amounts of CYP1A1 and NQO1 mRNA were measured by RT-PCR. As shown in Table 3, treatment of rats with 40 mg/kg bw of 3-ABA increased mRNA levels of CYP1A1 and NQO1 in the lung and NQO1 in the kidney.

3.2. The effect of 3-ABA pretreatment on activation of 3-NBA by rat pulmonary and renal cytosolic and microsomal fractions

Since NQO1 and CYP1A1, the enzymes relevant for 3-NBA and 3-ABA activation [12,13,20,21] were induced by 3-ABA, we investigated whether 3-ABA treatment increased the bioactivation of 3-ABA and 3-NBA by microsomal and cytosolic fractions. DNA adduct formation by 3-ABA and 3-NBA was measured by TLC ^{32}P-postlabelling.

Cytosolic samples from both organs were capable of reductively activating 3-NBA to species forming DNA adducts (see Fig. 3A for the renal cytosol). The DNA adduct pattern generated by 3-NBA consisted of a cluster of up to five adducts (spots 1−5 in Figure 3) essentially identical to that observed *in vivo* in rats and mice treated with 3-NBA [10,11,13,16,17], and in *in-vitro* incubations using human and rat hepatic cytosols [13] or microsomes [26]. TLC autoradiograms of ^{32}P-labelled DNA from control incubations carried out in parallel without cytosol, without DNA, or without 3-NBA were devoid of adduct spots in the region of interest (data not shown). Cochromatographic analysis of individual spots on HPLC confirmed that adduct spots 1−5 that are formed with rat pulmonary and renal cytosols are derived from 3-NBA by nitroreduction (data not shown). Three of these adducts were identified, and are 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone (dA-N^6-ABA; spot 1), 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-ABA; spot 3) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-ABA; spots 4 and 5) [15].

Pulmonary cytosolic samples from rats pretreated with 3-ABA (40 mg/kg bw) were more effective in the formation of 3-NBA-DNA adducts in the presence of NADPH, the cofactor of NQO1, than the pulmonary cytosol from untreated (control) rats (Fig. 4 and Supporting Table S1). The formation of 3-NBA-DNA adducts was 1.5-fold higher in incubations with cytosols of rats treated with 3-ABA relative to those of control rats. Addition of PAPS or acetyl-CoA, cofactors of SULTs and NATs, respectively, which enhance 3-NBA activation [13,20,24], to the incubations with induced cytosols had essentially the same stimulatory effects on 3-NBA adduct formation as in control cytosols. A 2.3- (with PAPS) and 20.3-fold (with acetyl-CoA) increase was observed in

comparison to the activity using NADPH alone. In renal cytosols similar effects were elicited by 3-ABA treatment, 3-NBA-DNA adduct levels were 1.2-fold higher than in incubations with cytosols from control kidneys. PAPS addition led to similar increases as in lung cytosolic incubations, but acetyl-CoA was even more effective, leading to ~30-fold higher levels of DNA adducts both in cytosols from 3-ABA treated rats and in those from control rats.

Contrary to the efficient 3-NBA activation by cytosolic reductases and conjugating enzymes, microsomal fractions isolated from either control or 3-ABA treated rat lungs or kidneys did not activate 3-NBA to DNA binding species.

3.3. The effect of 3-ABA pretreatment on activation of 3-ABA by rat pulmonary and renal cytosolic and microsomal fraction

In contrast to 3-NBA, 3-ABA was activated by lung and kidney microsomal fractions. CYP1A1 was found to play a major role in DNA adduct formation by 3-ABA in rat lungs and kidneys [21], because upon addition of NADPH, a cofactor of CYPs, all pulmonary and renal microsomal fractions were capable of oxidizing 3-ABA to form DNA adducts (see Fig. 3B for the renal microsomal fractions and Supporting Table S2). Kidney and lung microsomal fractions from rats treated with 3-ABA (40 mg/kg bw) exhibited 4.5- and 2.8-fold higher efficiencies to activate 3-ABA, than microsomal fractions from untreated rats, respectively (Fig. 5). Microsomal fractions from control and 3-ABA-treated rats also activated 3-ABA in the presence of arachidonic acid, a cofactor for COX-dependent oxidation [29-31] (Fig. 5 and Supporting Table S2). Autoradiograms of DNA digests from control incubations carried out in parallel either without microsomal fractions, without NADPH, without arachidonic acid, without DNA, or without 3-ABA were all devoid of adduct spots in the region of interest (data not shown).

In the presence of hydrogen peroxide, a cofactor for peroxidases, pulmonary and renal cytosols from control (uninduced) and 3-ABA-treated rats activated 3-ABA to form DNA adducts. In contrast to pulmonary cytosol, by which five 3-ABA-DNA adducts (spots 1-5 in Figure 3) were generated, only four 3-ABA-DNA adducts (spots 1-4 in Figure 3) were detected in the renal

cytosolic activation system. Treatment of rats with 3-ABA slightly stimulated the formation of 3-ABA-DNA adducts in cytosols from both organs (Fig 6 and Supporting Table S3). No adducts were detectable in controls without cytosols, without 3-ABA, or without hydrogen peroxide.

4. Discussion

3-ABA, the human metabolite of the ubiquitous environmental pollutant 3-NBA, has been detected in the urine of smoking and nonsmoking salt mine workers occupationally exposed to diesel emissions at similar concentrations (1-143 ng/24 h urine) to 1-aminopyrene (2-200 ng/24 h urine), the reduced metabolite of the most abundant nitro-PAH found in diesel exhaust [4]. The present study was undertaken to elucidate the potential of this chemical to induce the enzymes responsible for its own metabolic activation (CYP1A1) and that of the parent compound 3-NBA (NQO1) in the kidneys and lungs of rats. The rat was used as an experimental model, because the same enzymes activate 3-NBA and 3-ABA in human and rat livers [12-14,16,23,26] The results should therefore provide some indication of what might occur in extrahepatic tissues of humans exposed to this pollutant. Both enzymes are known to be inducible by several agents in animals and humans. Human and rat CYP1A1 is induced by many compounds, *e.g.* polychlorinated hydrocarbons such as 2,3,7,8-tetrachlorodibenzo[1,4]dioxine binding to the aryl hydrocarbon (Ah) receptor [32], and polycyclic aromatic hydrocarbons present in cigarette smoke [33,34]. NQO1 is inducible by a variety of agents with different mechanisms of action (see [35] for a review), including tumour promoters and hydrogen peroxide [36-38].

Expression levels and activities of NQO1 and CYP1A1 are also influenced by other factors, including several drugs, additional environmental chemicals and genetic polymorphisms [33,39,40]. So far two polymorphisms in the human *NQO1* gene have been found in the general population, one of which is associated with an increased risk of urothelial tumours [39] and paediatric leukaemia [41]. The polymorphic expression of CYP1A1 has been attributed to altered expression of the Ah receptor, the transcription factor that modulates its regulation, or the Ah receptor nuclear translocator (Arnt) protein, its associated transcription factor [33,34,42]. Moreover, the *CYP1A1* gene is genetically polymorphic [33,34]. So far, CYP1A1*2A, CYP1A1*2B and CYP1A1*4 polymorphisms have been found that might be associated with lung, oesophageal or breast cancer risk and with acute myeloid leukaemia [43-46]. Thus, genetic polymorphisms in *CYP1A* and *NQO1*

genes could be important determinants of a possible lung cancer risk from 3-NBA and its metabolite 3-ABA.

In the present study, we have shown that the expression of NQO1 and CYP1A1 proteins and their enzymatic activities was induced by 3-ABA in lungs of rats treated i.p. with a single dose of 0.4, 4 or 40 mg/kg bw of 3-ABA. This induction leads to an increase in enzyme activities and, thus, in the potential of these enzymes to activate 3-NBA in cytosols (by NQO1) and 3-ABA in microsomes (by CYP1A1) to form covalent DNA adducts, thereby enhancing the first step of their own bioactivation. 3-ABA also efficiently induced the expression of CYP1A1, but not NQO1, in the kidney. This is an interesting feature emphasizing the importance of 3-ABA for induction of its own oxidative activation in the kidney, through which it is excreted. The concentration of 3-ABA, which efficiently induced both enzymes in these extrahepatic organs, was very high, 4 and 40 mg/kg bw, and may not be relevant for human exposure levels. It was however a single dose, while humans are chronically exposed to low levels in certain work places in addition to cigarette smoke.

Generally, 3-ABA is a less efficient inducer of NQO1 or CYP1A1 in rat lung and kidney than typical strong inducers of these enzymes binding to Ah receptor such as 1-phenylazo-2-hydroxynaphtalene (Sudan I) [47-52] and ellipticine [53-60] or its parent nitro compound, 3-NBA [21]. Their potential to induce CYP1A and NQO1 enzymes in these extrahepatic rat tissues at doses similar to those of 3-ABA (0.4, 4 and 40 mg/kg bw) was up to 7-fold (Sudan I) [Stiborová et al., unpublished data] and 2-fold (3-NBA and ellipticine) [21,59,60] higher than that of 3-ABA. Likewise, the potency of these compounds to induce CYP1A1 was up to 6-fold higher compared to 3-ABA.

Induction of NQO1 and CYP1A1 mRNA levels by 3-ABA was lower than induction of proteins and activities of these enzymes. Similar discrepancies between induction of NQO1 and CYP1A mRNA and protein levels by 3-ABA were observed in rat livers [20] and also for other compounds as found by several authors [40,42,59,61-64]. It has been reported that some inducers might prolong half-lives of mRNAs, while others increase transcription. Moreover, half-lives of mRNAs are

usually much shorter than those of proteins [40,61,62]. A 24-h-treatment period was used in this study, because it was found to be an appropriate length of time to observe protein induction by several inducers [59,63-65]. The increase in CYP1A1 protein levels and enzyme activities reached a maximum at 12-20 h and then remained constant up to 24 h after treatment with a single dose of inducers before slowly dropping to background levels [59,63-65]. In contrast, CYP1A1 mRNA reached its highest level as early as 3-5 h [65-69].

Recently, we demonstrated that 3-ABA can be activated *in vitro* by *N*-oxidation by several peroxidases including MPO, LPO and COX (Fig. 1). Activation by peroxidase has also been found *in vivo*, mainly in kidneys and urinary bladders of 3-ABA-treated mice [14]. *N*-oxidation of 3-ABA finally leads to nitrenium ions that are responsible for the formation of 3-ABA-derived DNA adducts (Fig. 1). Whereas these ions are generated by CYP-mediated reactions *via N*-OH-3-ABA [12-14,19], peroxidases should catalyze their formation *via* an imino-derivative [14, 70-73]. In the present work we found that pulmonary and renal cytosols from rats treated with 3-ABA were more effective in activating 3-ABA to form DNA adducts in the presence of hydrogen peroxide than cytosols from control rats. The necessity for the presence of hydrogen peroxide indicates that 3-ABA activation might be associated with an increase in peroxidase activities in pulmonary and renal cytosols of 3-ABA-treated rats. However, the levels of DNA adducts generated by hydrogen peroxide-mediated 3-ABA activation were 2-fold lower than those by pulmonary and renal microsomes. Collectively, these results suggest that even though peroxidases might be involved in 3-ABA activation in these tissues, they play only a minor role.

3-ABA delivered i.p. is absorbed via the mesenteric veins and lymphatic system, and passes through the liver. Thus, its concentration and effect in this tissue should be higher than in the distal tissues such as lung and kidney. Indeed, the induction of NQO1 and CYP1A1 proteins and enzymatic activities in rat liver after identical i.p. treatment was up to 6- and 2-fold higher than in lungs and kidneys, respectively [20, present paper].

The activation of the parent nitro compound 3-NBA is much more effective by cytosolic reductases than by microsomal enzymes and 3-NBA is a more potent genotoxic compound than its metabolite 3-ABA. This is reflected in the levels of total DNA adducts generated in lung microsomes with NADPH from 3-ABA, which are not higher than those formed from 3-NBA in lung cytosol without added cofactor.

In conclusion, the results of the present study show, for the first time, that 3-ABA is capable of inducing NQO1 and CYP1A1 in lung and CYP1A1 in kidney of rats. These were found to be the predominant biotransforming enzymes involved in the metabolic activation of 3-NBA and 3-ABA, not only in these organs in rats [21], but also in the liver of both rats and humans [12,13,20]. 3-ABA also increased its own NADPH-dependent activation to DNA adducts in microsomal fractions from both organs, most effectively from kidneys. By such effects, 3-ABA exposure caused by its industrial and laboratory use or as a metabolite of 3-NBA, may lead to an enhancement of its own enzymatic activation, as well as that of 3-NBA, to reactive species forming DNA adducts, thereby enhancing the genotoxic and carcinogenic potential of both compounds.

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Table 1

Specific CYP1A1 activities [a] in kidney and lung microsomes of control and 3-ABA-treated rats

CYP activity	Control rats		3-ABA-treated rats [mg/kg bw]					
	Kidney	Lung	Kidney			Lung		
			0.4	4	40	0.4	4	40
EROD	9.4±0.4	4.6±0.5	20.0±0.5** (**2.1**)	39.6±0.9*** (**4.2**)	45.0±2.5*** (**4.8**)	5.1±0.5 (**1.1**)	6.4±0.4) * (**1.4**)	11.5±0.8** (**2.5**)
Sudan I oxidation	9.3±1.0	4.7±0.5	21.8±1.2** (**2.3**)	21.9±0.9** (**2.3**)	34.5±1.9*** (**3.7**)	4.7±0.5 (**1.0**)	6.2±0.3* (**1.3**)	10.9±0.7** (**2.3**)

[a]Each value (pmol of reaction product per min per mg protein) represents the mean ± S.E. of triplicate measurements. Numbers in parentheses represents the fold increase over the control activity caused by the pretreatment with 3-ABA (0.4, 4 or 40 mg/kg bw).

*Significantly different from controls: $p<0.05$ (Student's *t*-test).

**Significantly different from controls: $p<0.01$ (Student's *t*-test).

**Significantly different from controls: $p<0.001$ (Student's *t*-test).

Table 2

NQO1 specific activity in rat renal and pulmonary cytosol

	Control rats	3-ABA-treated rats [mg/kg bw] 0.4	4	40
Kidney	0.09 ± 0. 01	0.08 ± 0. 01 (**0.9**)	0.10 ± 0. 01 (**1.1**)	0.10 ± 0. 01 (**1.1**)
Lung	0.05 ± 0.01	0.06 ± 0.01* (**1.2**)	0.07 ± 0.01* (**1.4**)	0.08 ± 0.01** (**1.6**)

The results (units) are averages and S.E. of five parallel measurements. Enzyme activities with menadione as a substrate was assayed as described in Materials and Methods. One unit of NQO1 reduces 1 μmol of NADPH per min/mg protein at 37°C. Numbers in parentheses represents the fold increase over the control activity caused by the pretreatment with 3-ABA (0.4, 4 or 40 mg/kg bw).

*Significantly different from controls: $p<0.05$ (Student's *t*-test).

**Significantly different from controls: $p<0.01$ (Student's *t*-test).

Table 3

Expression of mRNA of CYP1A1 and NQO1

	CYP1A1		NQO1	
	Δc_T[a]	Fold Change[b]	Δc_T	Fold Change
Control rats				
Kidney	4.45 ± 0.36	-	9.37 ± 0.67	-
Lung	10.48 ± 0.16	-	5.44 ± 0.34	-
3-ABA-treated rats				
Kidney	4.49 ± 0.06	**1.0**	9.07 ± 0.22*	**1.2**
Lung	10.02 ± 0.10**	**1.4**	4.97 ± 0.11**	**1.4**

[a]Results shown are mean ± S.D. from data found for three rats (control and treated with 40 mg/kg bw of 3-ABA).

[b]The induction of mRNA expression of studied target genes in pretreated animals (fold change) was evaluated as $2^{-(\Delta\Delta cT)}$.

*Significantly different from controls: $p<0.05$ (Student's *t*-test).

**Significantly different from controls: $p<0.01$ (Student's *t*-test)

Legends to Figures

Fig. 1. Pathways of metabolic activation and DNA adduct formation of 3-NBA and 3-ABA. See text for details. NQO1, NAD(P)H:quinone oxidoreductase; NAT, *N*,*O*-acetyltransferases; SULT, sulfotransferase; COX-1, cyclooxygenase 1; CYP, cytochrome P450; LPO, lactoperoxidase; MPO, myeloperoxidase; POR, NADPH:cytochrome P450 oxidoreductase; R = $-COCH_3$ or $-SO_3H$.

Fig. 2. Induction of CYP1A1 (A,B) and NQO1 (C,D) in kidneys (A,C) and lungs (B,D) of rats treated with 0.4, 4 or 40 mg/kg bw of 3-ABA determined by Western blots as described in Material and methods. Immunoblots of microsomal CYP1A1 and cytosolic NQO1 from untreated and 3-NBA-treated rats stained with antibody against rat CYP1A1 and human NQO1. Values represent mean ± S.D. obtained from lungs and kidneys of three rats (n=3). Values significantly different from control: $*p<0.05$, $**p<0.01$, $***p<0.001$ (Student's *t*-test).

Fig. 3. Autoradiographic profiles of DNA adducts generated (A) by 3-NBA after its activation with cytosols isolated from lungs of rats treated with 40 mg/kg bw of 3-ABA and NADPH, and (B) by 3-ABA after its activation with microsomes isolated from kidneys of rats treated with 40 mg/kg bw of 3-ABA and NADPH by using the butanol enrichment version of the ^{32}P-postlabelling assay. Spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

Fig. 4. DNA adduct formation by 3-NBA activated with cytosols isolated from kidneys and lungs of rats, control (uninduced) or pretreated with 40 mg/kg bw of 3-ABA, using different cofactors. See text for details. F = fold increase in DNA adduct levels in cytosols from 3-ABA-treated rats compared to control (uninduced) rats. Mean ± S.E. shown in the figure represent total levels of DNA adducts (RAL, relative adduct labelling) of three determinations. $*p<0.05$, $**p<0.01$ (Student's *t*-test).

Fig. 5. DNA adduct formation by 3-ABA activated with microsomes isolated from kidneys and lungs of rats, control (uninduced) or pretreated with 40 mg/kg bw of 3-ABA, using different cofactors. F = fold increase in DNA adducts levels in microsomes from 3-ABA-treated rats compared to control (uninduced) rats. Mean ± S.E. shown in the figure represent total DNA adduct levels (RAL, relative adduct labelling) of three determinations. None = without cofactor; AA = arachidonic acid. ND = not detected. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's *t*-test).

Fig. 6. DNA adduct formation by 3-ABA activated with cytosols isolated from kidneys and lungs of rats, control (uninduced) or pretreated with 40 mg/kg bw of 3-ABA, using hydrogen peroxide as a cofactor. Mean ± S.E. shown in the figure represent total DNA adduct levels (RAL, relative adduct labelling) of three determinations. None = without cofactor; ND = not detected. *$p<0.05$ (Student's *t*-test).

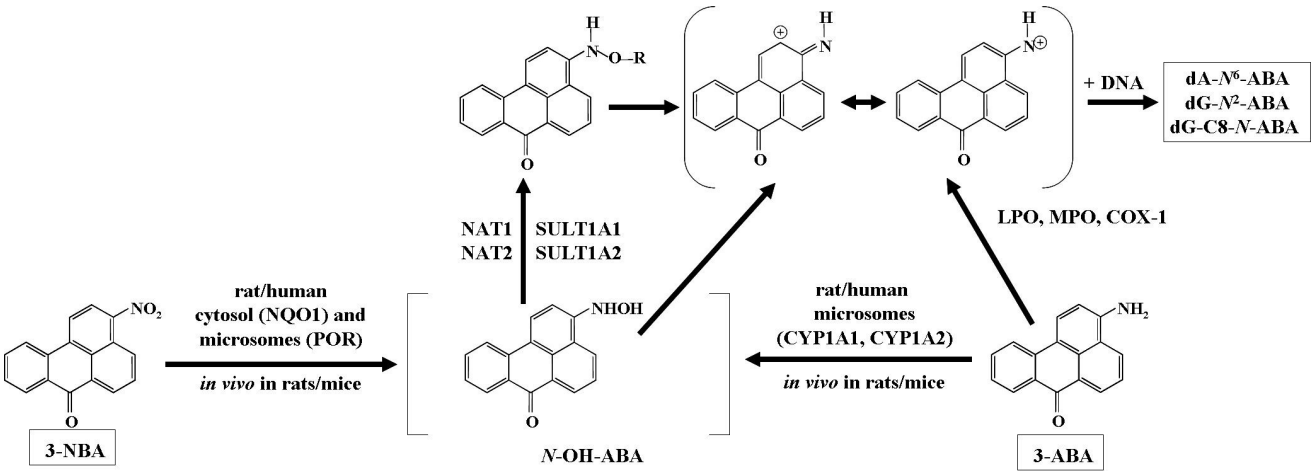
NO2
3-NBA
rat/human
cytosol (NQO1) and
microsomes (POR)
in vivo in rats/mice
NHOH
N-OH-ABA
NAT1
NAT2
SULT1A1
SULT1A2
O–R
rat/human
microsomes
(CYP1A1, CYP1A2)
in vivo in rats/mice
NH2
3-ABA
LPO, MPO, COX-1
+ DNA
dA-N6-ABA
dG-N2-ABA
dG-C8-N-ABA

Figure 1

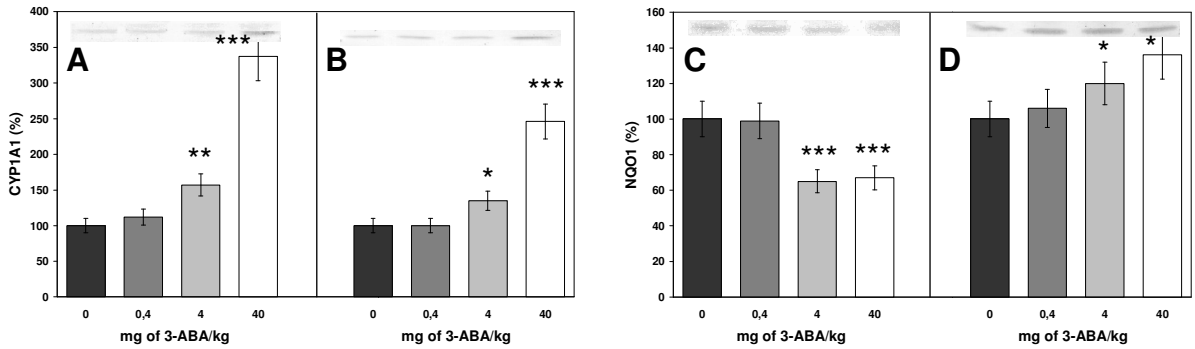

Figure 2

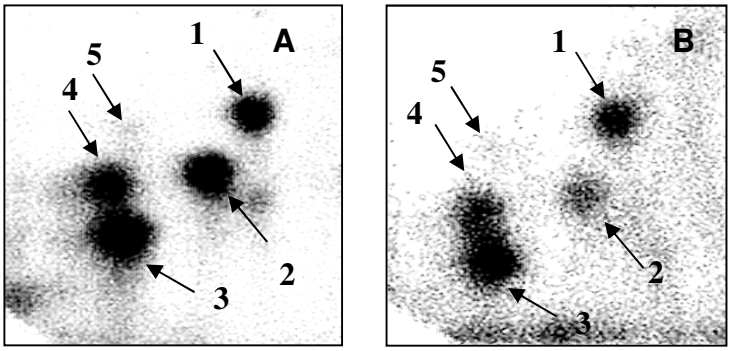

Figure 3

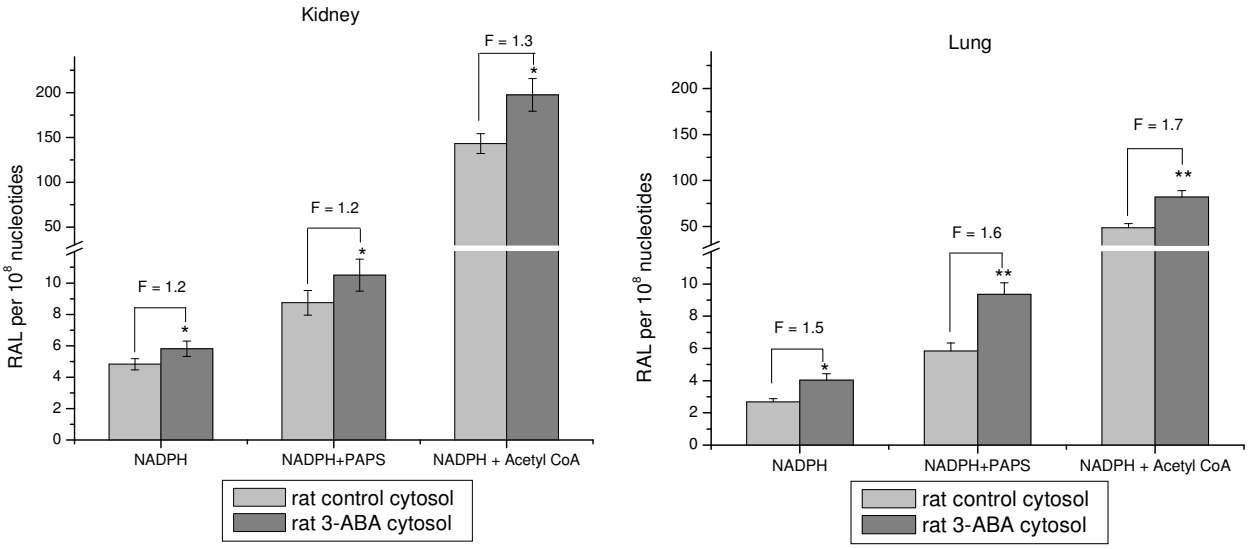
Kidney
RAL per 10^8 nucleotides
F = 1.2
F = 1.2
F = 1.3
*
*
*
NADPH
NADPH+PAPS
NADPH + Acetyl CoA
rat control cytosol
rat 3-ABA cytosol
Lung
RAL per 10^8 nucleotides
F = 1.5
F = 1.6
F = 1.7
*
**
**
NADPH
NADPH+PAPS
NADPH + Acetyl CoA
rat control cytosol
rat 3-ABA cytosol

Figure 4

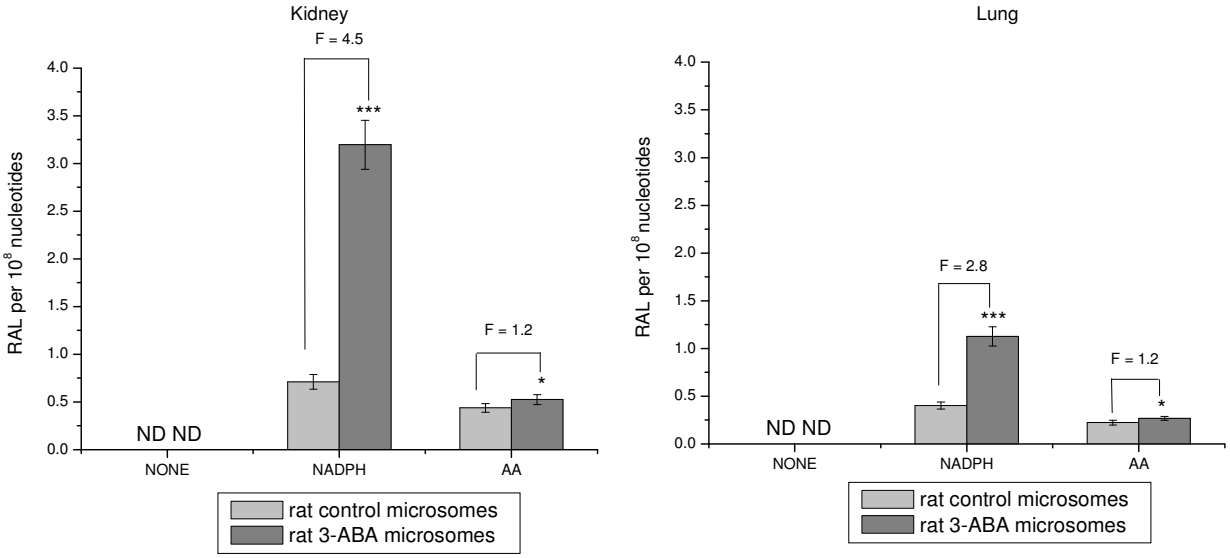
Kidney
F = 4.5
***
F = 1.2
*
ND ND
RAL per 10^8 nucleotides
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NONE
NADPH
AA
rat control microsomes
rat 3-ABA microsomes
Lung
F = 2.8
***
F = 1.2
*
ND ND
RAL per 10^8 nucleotides
NONE
NADPH
AA
rat control microsomes
rat 3-ABA microsomes

Figure 5

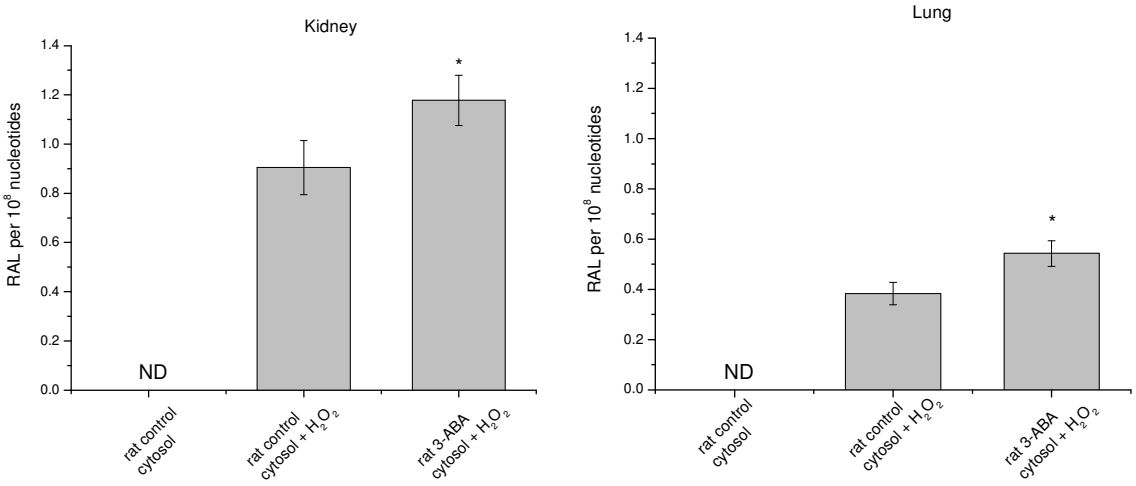
Kidney
RAL per 10^8 nucleotides
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ND
*
rat control cytosol
rat control cytosol + H2O2
rat 3-ABA cytosol + H2O2
Lung
RAL per 10^8 nucleotides
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ND
*
rat control cytosol
rat control cytosol + H2O2
rat 3-ABA cytosol + H2O2

Figure 6

Supplementary data

Supporting Table S1.

a) Effect of enzyme cofactors on DNA adduct formation by 3-ABA in rat kidney cytosol from control rats or rats treated with 40 mg 3-ABA/kg bw

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced kidney cytosol without cofactor	**0.148** ± 0.013	**0.310** ± 0.289	**0.823** ± 0.754	**0.449** ± 0.398	**0.066** ± 0.010	**1.796** ± 0.154
uninduced kidney cytosol + NADPH	**0.467** ± 0.051	**0.698** ± 0.061	**1.797** ± 0.142	**1.483** ± 0.132	**0.392** ± 0.034	**4.82** ± 0.360
uninduced kidney cytosol + NADPH + PAPS	**0.749** ± 0.064	**1.369** ± 0.327	**2.978** ± 0.234	**3.061** ± 0.300	**0.583** ± 0.052	**8.74** ± 0.782
uninduced kidney cytosol + NADPH + acetylCoA	**12.80** ± 1.001	**38.63** ± 3.123	**43.65** ± 4.176	**46.61** ± 4.101	**1.38** ± 0.151	**143.08** ± 11.201
3-ABA kidney cytosol + NADPH	**0.561** ± 0.102	**0.838** ± 0.140	**2.156** ± 0.189	**1.779** ± 0.152	**0.470** ± 0.038	**5.804** ± 0.487
3-ABA kidney cytosol + NADPH + PAPS	**0.899** ± 0.087	**1.643** ± 0.101	**3.584** ± 0.265	**3.673** ± 0.301	**0.699** ± 0.098	**10.498** ± 1.021
3-ABA kidney cytosol + NADPH + acetylCoA	**17.92** ± 1.24	**54.082** ± 4.254	**61.11** ± 5.877	**62.254** ± 5.989	**1.932** ± 0.241	**197.298** ± 18.142

b) Effect of enzyme cofactors on DNA adduct formation by 3-ABA in rat lung cytosol from control rats or rats treated with 40 mg 3-ABA/kg bw

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced lung cytosol without cofactor	**0.084** ± 0.011	**0.054** ± 0.009	**0.151** ± 0.012	**0.075** ± 0.010	**0.074** ± 0.010	**0.443** ± 0.034
uninduced lung cytosol + NADPH	**0.189** ± 0.015	**0.298** ± 0.021	**1.210** ± 0.102	**0.882** ± 0.068	**0.110** ± 0.011	**2.688** ± 0.190
uninduced lung cytosol + NADPH + PAPS	**0.511** ± 0.048	**0.977** ± 0.101	**1.989** ± 0.132	**1.989** ± 0.165	**0.386** ± 0.030	**5.852** ± 0.476
uninduced lung cytosol + NADPH + acetylCoA	**5.13** ± 0.498	**7.71** ± 0.671	**13.88** ± 1.151	**17.826** ± 1.482	**3.712** ± 0.301	**48.656** ± 4.426
3-ABA lung cytosol + NADPH	**0.284** ± 0.020	**0.447** ± 0.032	**1.815** ± 0.121	**1.323** ± 0.087	**0.165** ± 0.081	**4.034** ± 0.395
3-ABA lung cytosol + NADPH + PAPS	**0.818** ± 0.067	**1.563** ± 0.078	**3.182** ± 0.201	**3.182** ± 0.198	**0.618** ± 0.061	**9.363** ± 0.712
3-ABA lung cytosol + NADPH + acetylCoA	**8.208** ± 0.802	**12.336** ± 1.214	**22.208** ± 2.010	**28.522** ± 2.351	**10.740** ± 0.910	**82.014** ± 7.132

[a] Mean ± S.E. (RAL, relative adduct labelling) of triplicate *in-vitro* incubations; spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

Supporting Table S2.

a) DNA adduct formation by 3-ABA activated by kidney microsomes of rats either uninduced or induced with 40 mg 3-ABA/kg bw

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced without cofactor	ND[b]	ND	ND	ND	ND	ND
uninduced s + NADPH	**0.067** ± 0.007	**0.087** ± 0.009	**0.377** ± 0.032	**0.154** ± 0.014	**0.025** ± 0.003	**0.710** ± 0.078
uninduced + arachidonic acid	**0.055** ± 0.006	**0.096** ± 0.010	**0.197** ± 0.018	**0.088** ± 0.003	ND	**0.436** ± 0.045
3-ABA microsomes without cofactor	ND	ND	ND	ND	ND	ND
3-ABA microsomes + NADPH	**0.302** ± 0.019	**0.392** ± 0.030	**1.697** ± 0.010	**0.693**± 0.0541	**0.112** ± 0.010	**3.196** ± 0.257
3-ABA microsomes + arachidonic acid	**0.066** ± 0.010	**0.115** ± 0.011	**0.237** ± 0.021	**0.106** ± 0.010	ND	**0.524** ± 0.051

b) DNA adduct formation by 3-ABA activated by lung microsomes of rats either uninduced or induced with 40 mg 3-ABA/kg bw

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced lung microsomes without cofactor	ND[b]	ND	ND	ND	ND	ND
uninduced lung microsomes + NADPH	**0.072** ± 0.008	**0.062** ± 0.006	**0.154** ± 0.012	**0.059** ± 0.004	**0.055** ± 0.005	**0.402** ± 0.037
uninduced lung microsomes + arachidonic acid	**0.035** ± 0.004	**0.035** ± 0.004	**0.101** ± 0.010	**0.026** ± 0.003	**0.026** ± 0.003	**0.223** ± 0.025
3-ABA lung microsomes without cofactor	ND	ND	ND	ND	ND	ND
3-ABA lung microsomes + NADPH	**0.202** ± 0.011	**0.174** ± 0.013	**0.431** ± 0.035	**0.165**± 0.015	**0.154** ± 0.012	**1.126** ± 0.101
3-ABA lung microsomes + arachidonic acid	**0.042** ± 0.004	**0.043** ± 0.004	**0.121** ± 0.011	**0.032** ± 0.011	**0.031** ± 0.003	**0.269** ± 0.020

[a] Mean ± S.E. (RAL, relative adduct labelling) of triplicate *in-vitro* incubations; spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

[b] ND = not detected.

Supporting Table S3.

a) DNA adduct formation by 3-ABA activated by kidney cytosols of rats either uninduced or induced with 40 mg 3-ABA/kg bw

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced cytosol	ND[b]	ND	ND	ND	ND	ND
uninduced cytosol + H_2O_2	**0.237**± 0.023	**0.233** ± 0.023	**0.272** ± 0.029	**0.163** ± 0.014	ND	**0.905** ± 0.110
3-ABA cytosol + H_2O_2	**0.309** ± 0.032	**0.303** ± 0.025	**0.354** ± 0.031	**0.212**± 0.020	ND	**1.178** ± 0.102

b) DNA adduct formation by 3-ABA activated by lung cytosols of rats either uninduced or induced with 40 mg 3-ABA/kg bw

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced cytosol	ND[b]	ND	ND	ND	ND	ND
uninduced cytosol + H_2O_2	**0.057**± 0.007	**0.072** ± 0.008	**0.128** ± 0.015	**0.066** ± 0.008	**0.060** ± 0.007	**0.383** ± 0.045
3-ABA cytosol + H_2O_2	**0.090** ± 0.010	**0.087** ± 0.010	**0.166**± 0.011	**0.110** ± 0.012	**0.090** ± 0.010	**0.543** ± 0.051

[a] Mean ± S.E. (RAL, relative adduct labelling) of triplicate *in-vitro* incubations; spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

[b] ND = not detected.